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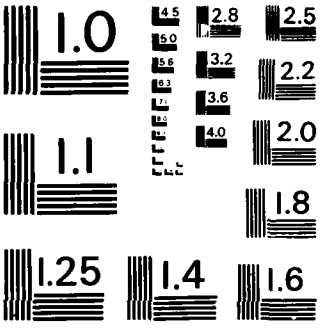

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DYE FLUORESCENCE ANALYSIS FROM BACTERIAL METABOLISM

by A. Peter Snyder
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RESEARCH DIVISION
CB DETECTION & ALARMS DIVISION

April 1984

US Army Armament, Munitions & Chemical Command
Aberdeen Proving Ground, Maryland 21010

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1. REPORT NUMBER CRDC-TR-84013	2. GOVT ACCESSION NO. AD A142 667	3. RECIPIENT'S CATALOG NUMBER
4. TITLE *(and Subtitle)* DYE . ORESCENCE ANALYSIS FROM BACTE AL METABOLISM		5. TYPE OF REPORT & PERIOD COVERED Technical Report December 1982-March 1983 6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) A. Peter Snyder David B. Greenberg Theresa T. Wang		8. CONTRACT OR GRANT NUMBER(s)
9. PERFORMING ORGANIZATION NAME AND ADDRESS Commander Chemical Research and Development Center ATTN: DRSMC-CLB-R (A) Aberdeen Proving Ground, Maryland 21010		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 1L161101A91A
11. CONTROLLING OFFICE NAME AND ADDRESS Commander Chemical Research and Development Center ATTN: DRSMC-CLJ-IR (A) Aberdeen Proving Ground, Maryland 21010		12. REPORT DATE April 1984 13. NUMBER OF PAGES 20
14. MONITORING AGENCY NAME & ADDRESS*(if different from Controlling Office)*		15. SECURITY CLASS. *(of this report)* UNCLASSIFIED 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE NA

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17. DISTRIBUTION STATEMENT *(of the abstract entered in Block 20, if different from Report)*

18. SUPPLEMENTARY NOTES

19. KEY WORDS *(Continue on reverse side if necessary and identify by block number)*

Induced fluorescence
Diacetylfluorescein
Bacterial metabolism
Fluorescent product
Lipase

20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

A method based upon induced fluorescence is proposed for the rapid detection and characterization of viable microorganisms. In this technique, a nonfluorescing dye is metabolized intracellularly by an organism through an enzyme-specific reaction. This produces a fluorescent product which when desorbed can be detected. The technique has been applied successfully to the microorganism

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20. ABSTRACT (Contd)

Bacillus globigii through the enzyme lipase and the nonfluorescing dye diacetylfluorescein. By relating the effect of bacterial and substrate activity to the initial rate of fluorescence generation, standard curves have been obtained from which both age and bacterial concentration can be estimated.

PREFACE

The work described in this report was authorized under Project 1L161101A91A, CB Detection and Alarms. This work was started in December 1982 and completed in March 1983.

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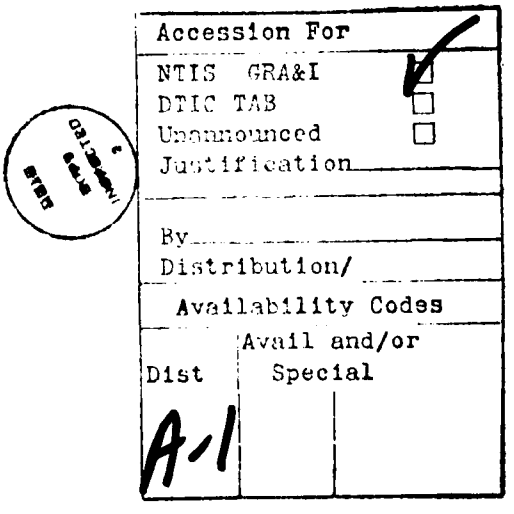

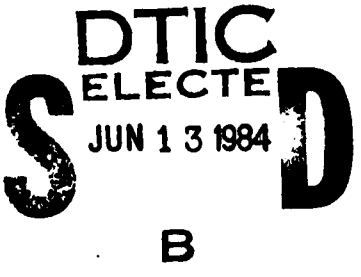

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DYE FLUORESCENCE ANALYSIS FROM BACTERIAL METABOLISM

1. INTRODUCTION

A methodology for rapid detection and identification of microorganisms has long been of concern to the medical, pharmaceutical, and food processing fields. Because of this sustained interest, the area has remained active and has seen significant advances over the classical time-consuming protocols of standard plate counting, membrane filtration, or the multiple-tube fermentation procedures. However, even today these techniques can require as much as 1 to 3 days; hence, continued research is still indicated before a rapid, generalized microbial characterization scheme is to be realized.

Various earlier developments have focused upon techniques in immunofluorescence,[1-6] enzyme amplification,[7,8] light detection and ranging (LIDAR),[9] mass spectrometry,[10] bacteriophage lysis,[11-13] computer assisted probabilistic methods,[14-18] the recently developing field of gel ferrography[19,*] and the double fluorescent DNA staining method coupled to flow cytometry.[20] Each of these techniques is limited by disadvantages such as laborious and time-consuming laboratory preparation and sample handling, long observation times, and nonspecificity with respect to bacterial characterization and subsequent identification.

The present investigation, which we believe to offer the possibilities of speed, sensitivity, and specie identification to the process of microorganism detection is embraced in the following equation:

$$\underset{\text{substrate}}{\text{nonfluorescent}} + \underset{\text{(in bacteria)}}{\text{enzyme}} \xrightarrow[\text{cofactors}]{\text{supplied}} \underset{\text{product}}{\text{fluorescent}} + \text{metabolites} \quad (1)$$

This method combines several attractive experimental features. These include

(a) exploitation of the particular enzymatic/metabolic machinery of the cell by an added substrate in which a predetermined reaction takes place,

(b) the product fluorescence which can be used to indicate a positive cellular response, and

(c) rapid data collection and analysis under 30 minutes.

Equation 1 presents various modes of biochemical strategy in order to uniquely characterize a given bacterium or small group of bacteria. The technique pinpoints the identity of a particular enzyme that is indigenous to the bacteria themselves through several

*J. K. Marquis, A. P. Russell, and V. C. Westcott. Boston University Medical Center. Personal communication, 1983.

experimental avenues. In one instance, for the same bacterial enzyme a series of chemically similar substrates (e.g., esters) can be utilized to generate unique reaction rates and, thus, associated product fluorescence. Another scheme involves sequentially using a series of substrates in order to trigger the various potential enzymatic reactions that can occur within the bacterium. The present experimental format exploits the former procedure.

The first-order nature of the experimental fluorescence-producing chemistry in this work suggests the Michaelis-Menten data analysis approach as most attractive. Precedence for applying Michaelis-Menten kinetics to enzymatic processes *in vivo* (i.e., reactions occurring in the intact, living cell) has been previously documented in the literature,[21,22] although the theory, strictly speaking, was initially developed for enzymatic processes occurring *in vitro*.

2. EXPERIMENTAL METHODS AND MATERIALS

2.1 Bacterial Preparation and Handling.

Bacillus subtilis var *niger* (*Bacillus globigii*, BG), the test organism, was grown on a nutrient agar slant for 24 hours at 37°C and stored at 4°C. One loopful of the inoculum culture from the slant was spread evenly over the agar surface of the nutrient agar plates with a sterile glass rod. After 16 to 18 hours incubation at 37°C, the growth layer was scraped from the agar by adding 10 ml of sterile 0.1 M Tris, 0.85% NaCl, pH 8.0, in each plate. The cell suspension was diluted to 75 ml with the buffer and was stored at 4°C. Viable cell counts of the bacterial samples in the first set of experiments (*vide infra*) were made by plating serial dilutions using sterile distilled water as diluting blank , tryptose agar as plating agar, 37°C as incubation temperature, and 36 hours as incubation time.

Nutrient agar and tryptose agar were purchased from DIFCO Laboratories, Detroit, Michigan.

2.2 Bacterial Assays.

The specific reaction examined was the hydrolysis of the substrate diacetylfluorescein (5.0×10^{-5}M) by microbial lipase. Normally, methylcellosolve[23-25] and acetone[27] are the solvents used to dissolve diacetylfluorescein with subsequent buffer dilution; however, we found early in our work that acetone provides approximately twice the activity as methylcellosolve. A typical assay solution consisted of 2.0 ml of the dye solution at a prepared dye concentration[23-26] and 0.1 ml of the bacterial suspension added at the beginning of the assay. A Farrand fluorimeter, Model MK-2, was used to excite diacetylfluorescein at 469 nm and to monitor the emission at 519 nm with excitation and emission slits of 1.0 and 2.5 nm, respectively.

3. RESULTS AND DISCUSSION

Two sets of experiments were conducted over a 1-month period using the same suspension in order to observe the effect of bacterial age on the significant parameters. In one study in which the substrate concentration was held constant, the bacterial concentration was varied

and the initial reaction rate was determined via the tangent to the initial portion of the fluorescence generation curve. Figures 1 and 2 reflect these results. The plot presented in Figure 1 shows a marked contrast in the differentiation of the hydrolysis rates of bacterial diacetylfluorescein above 5.0 x 10^4 cells/ml, which correlates well with the data of Pinteno and Findl[27] as shown in Figure 3. Their work made use of the enzymatic hydrolysis of fluorescein-di-β-galactopyranoside with *Escherichia coli* neotype. The normalized comparison between the two studies suggest similar kinetics.

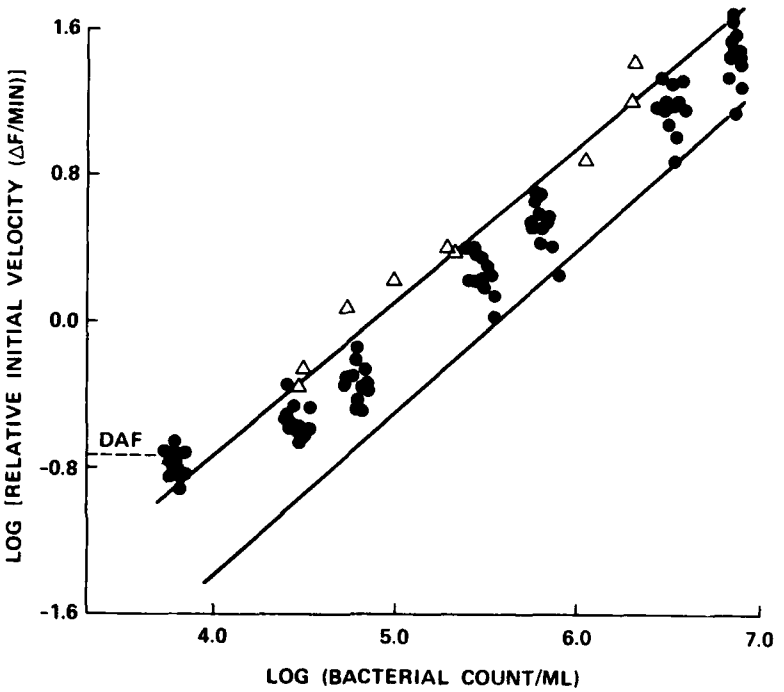

Figure 1. Log Plot of the Concentration of an Aging *B. globigii* (BG) Suspension vs the Relative Diacetylfluorescein (DAF) Hydrolysis Rate

The lines represent the maximum and minimum experimentally determined limits. The points designated (Δ) represent experiments on a separate 1 to 3 day old BG suspension. The point at which the rate of spontaneous DAF hydrolysis and the bacterial detection limit of sensitivity coincides is noted at the lower left.

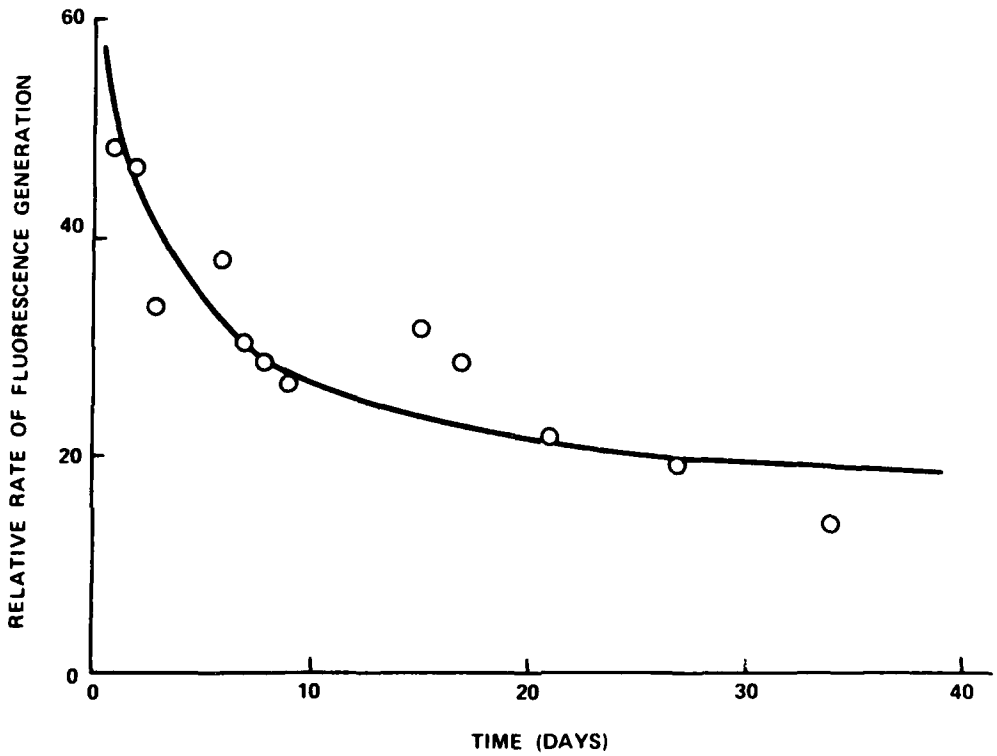

Figure 2. The Effect of Age vs the Relative Diacetylfluorescein Hydrolysis Rate at a Viable Bacteria Concentration (*B. globigii*) of Approximately 5.50×10^6 cells/ml

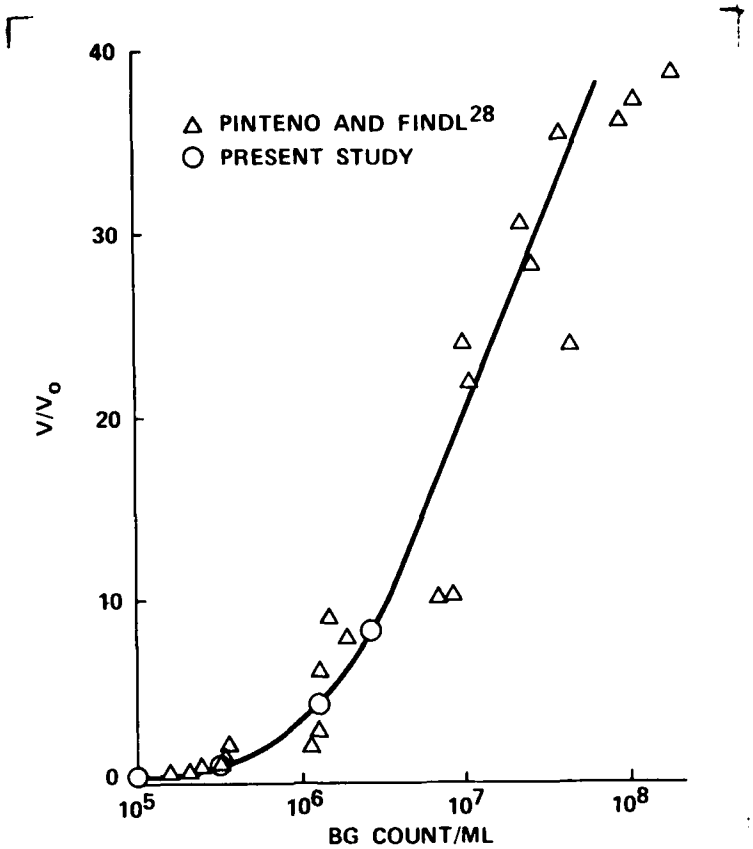

Figure 3. A Comparison of Fluorescence Production vs Microorganism Concentration Between the Work of Pinteno and Findl[28] (*E. coli*) and This Study (*B. subtilis*)

Bacterial age was not addressed in the Pinteno and Findl report; whereas, The BG from this work was taken from 1- to 2-day old suspension data.

As the bacterial suspension aged, a pronounced sensitivity in age delineation is observed above 5.0 x 10^4 cells/ml. Figure 2 shows a plot of age vs the relative hydrolysis rate of diacetylfluorescein for a concentration of approximately 5.0 x 10^6 cells/ml. An apparently exponential decay of the fluorescein emission is observed. This effect is interesting in that, despite the decrease in metabolic activity of the cell suspension with time, the viable *B. subtilis* population, as shown in Figure 1, appears to remain constant. Possible explanations of this anomaly include (a) genetic repression of the lipase-producing cellular machinery, (b) intracellular degradation of the metabolic machinery, and (c) cell wall modification due to aging. Therefore, the log plot of Figure 1 becomes generally useful for determining both the bacterial concentration and age. For the present buffer and detection system, Figure 1 shows clearly that the limit of bacterial concentration, in which the signal-to-noise ratio is twice that of the spontaneous hydrolysis of diacetylfluorescein, is 3.0 x 10^4 cells/ml, approximately. If this graph is to be a generally useful and predictive tool for a particular bacterial concentration and age, it must be independent of

microbial suspensions for that same microorganism. This thesis is apparently supported by a separate B. *subtilis* suspension of the same age (1 to 3 days) whose data (Δ) is superimposed upon the graph of the figure. These results indicate that for a sample analysis one need only measure the relative velocity of an enzymatic *in vivo* reaction in order to obtain the bacterial concentration from the previously prepared standard curve. At the same time, a rough bacterial age can be estimated.

In the second series of experiments, diacetylfluorescein concentrations were varied from 2.4×10^{-5}M to 4.8×10^{-8}M while, again, monitoring the rate of reaction. The viable B. *subtilis* bacterial concentration remained constant during the course of this study; hence a standard of 5.5×10^{6} cells/ml assay solution was used. Because the intracellular hydrolysis of diacetylfluorescein obeys Michaelis-Menten kinetics,[28] the enzyme parameter, K_M was able to be evaluated for each daily set of runs from Hanes-Woolf plots.[28,29] Since it was first postulated and then observed (see Figure 1) that lipase activity on diacetylfluorescein decreases as the bacterial suspension ages, the use of the Michaelis-Menten parameter was included in our investigation. Over the 1-month course of this study, relative K_M values varied only modestly (0.6 to 1.6×10^{-5}M) in a random fashion, with an average value of 1.17×10^{-5}M. This is to be expected because the Michaelis-Menten theory dictates that K_M is independent of the enzyme concentration;[28] therefore, the data suggest that the parameter might be used to more completely identify a specific bacterial-enzyme system. The data representing the separate BG suspension superimposed on Figure 1 yielded a K_M value of 1.38×10^{-5}M, which is in reasonable agreement with the value cited above. It is of further interest to note that in other studies of intracellular diacetylfluorescein hydrolysis, K_M values of 3.6×10^{-5}M for fibroblast L cells,[22] 2.9×10^{-6}M for cultured mouse lymphoma cells,[21] and average intracellular fluorescein concentrations of 4.4×10^{-5}M and 2×10^{-5}M for leukemic and normal mouse lymphocytes,[30] respectively, were reported. To the best of our knowledge, these represent the only quantitative Michaelis-Menten analyses of experiments with living mammalian and bacterial cells. K_M values of 3.6×10^{-5}M and 7.0×10^{-6}M were reported for the cell-free extracts of the cultured mouse lymphoma cells mentioned above and an *in vitro* solution of porcine pancreas lipase,[23,31] respectively. At a K_M value of 1.17×10^{-5}M, the B. *subtilis* hydrolyzed diacetylfluorescein is within an order of magnitude of that found in the mammalian cells and the *in vitro* lipase experiment.

4. CONCLUSIONS

These encouraging early results suggest that further study concerning this technique as a valid approach to microorganism determination is warranted. From this work there emerges various parameters that appear to be useful in the determination of both concentration and age of a bacterium. These include the observed reaction rate and the Michaelis-Menten constant. In addition, the method suggests that sequential substrate usage could permit further refining of the process of uniquely identifying microorganisms.

The characterization of a mixed bacteria sample, however, is significantly more germane. By the very nature of enzyme reactions, the substrates themselves shed light on the kind of bacteria present. Thus, a quick antibiotic screen and/or biochromatographic techniques utilizing different retention times for different microorganisms* could also be used in bacterial separation and determination. Greater bacterial resolution could be achieved by coupling a low-power source of laser excitation with a microfluorimeter in a microscopic stage system. This is the subject of continuing work by the authors.

*D. B. Greenberg, University of Cincinnati, and G. Janauer, State Universtiy of NY at Binghamton. 1983.

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LITERATURE CITED

1. Halmann, M., Velan, B., and Sery, T. Appl. Environ. Microbiol. *34*, 473 (1977).

2. Drow, D. L., Maki, D. G., and Manning, D. D. J. Clin. Microbiol. *10*, 442 (1979).

3. Wolters, G., Kuijpers, L. P., Kacaki, J., and Schuurs, H.W.M. J. Infect. Dis. *136*, s311 (1977).

4. Goldman, M. Ann. N. Y. Acad. Sci. *177*, 439 (1971).

5. Danielsson, D., Nathorst-Windahl, G., and Salden, T. Ibid., 23.

6. Pavlova, M. T., Beauvais, E., Brezenski, F. T., and Litsky, W. Appl. Microbiol. *23*, 571 (1972).

7. Hinsberg, W. D., Milby, K. H., Lidofsky, S. D., and Zare, R. N. Proc. SPIE. J. A. Gelbwachs, ed. *286*, 132 (1981).

8. Imsaka, T., and Zare, K. N. Anal. Chem. *51*, 2082 (1979).

9. Betz, H., Buhay, H., Fenters, J., and Vana, S. ARCSL-CR-81039. May 1981. UNCLASSIFIED Report.

10. Anhalt, J. P. Anal. Chem. *47*, 219 (1975).

11. Kellenberger, G. and Kellenberger, E. Virology *3*, 275 (1957).

12. Pijper, A. J. Path. Bact. *57*, 1 (1945).

13. Smith, P. B., and Cherry, W. B. J. Inf. Dis. *96*, 34 (1955).

14. Feltham, R.K.A., and Sneath, P.H.A. J. Gen. Microbiol. *128*, 713 (1982).

15. Kelley, R. W., and Kellogg, S. T. Appl. Environ. Microbiol. *35*, 507 (1978).

16. Smith, P. B., Gavin, T. L., Isenberg, H. D., Sonnenwirth, A., Taylor, W. I., Washington, J. A. II, and Balows, A. J. Clin. Microbiol. *8*, 657 (1978).

17. Friedman, R. B., and MacLowry, J. Appl. Microbiol. *26*, 314 (1973).

18. Friedman, R. B., Bruce, D., MacLowry, J., and Brenner, V. Am. J. Clin. Pathol. *60*, 395 (1973).

19. Russell, A. P., DeMaria, A., Johns, M., and Westcott, V. C. J. Tribology (1983).

20. Van Dilla, M. A., Langlois, R. G., Pinkel, D., Yajko, D., and Hadley, W. K. Science *220*, 620 (1983).

21. Rotman, B., and Papermaster, B. W. Proc. Nat'l. Acad. Sci. *55*, 135 (1966).

22. Sernetz, M. Fluorescence Techniques in Cell Biology, p. 243. Springer-Verlag, Berlin. 1973.

23. Guilbault, G. G., and Hieserman, J. Anal. Chem. *41*, 2006 (1969).

24. Kramer, D. N., and Guilbault, G. G. Ibid., *35*, 588 (1963).

25. Guilbault, G. G., and Kramer, D. N. Ibid., *36*, 409 (1964).

26. Mitz, M. A., Blanchard, G. C., and Deacon, T. E. U. S. Army Biological Laboratories. DA-18-064-CML-2842. July 1963. UNCLASSIFIED Report.

27. Pinteno, F., and Findl, E. Naval Research Contract N00014-78-C-0713. February 1981. UNCLASSIFIED Report.

28. Segel, I. H., ed. Biochemical Calucations. 2nd Ed. Wiley-Interscience. 1975.

29. Segel, I. H., ed. Enzyme Kinetics. Chapter 4. Wiley-Interscience. 1975.

30. Szollosi, J., Kertai, P., Somogyi, B., and Damjanovich, S. J. Histochem. Cytochem. *29*, 503 (1981).

31. Sherman, W. R., and Stanfield, E. F. Biochem. J. *102*, 905 (1967).

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